A Bitter Principle of Tomato Seeds

Isolation and Structure of a New Furostanol Saponin

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From the Bitter tasting seeds of the tomato (Lycopersicon esculentum Miller), a bitter principle (TFI) was isolated. The chemical structure was established as 5β-furostane-3β,22,26-triol-3-O-β-D-glucopyranosyl (1→2)-β-D-glucopyranosyl (1→4)-β-D-galactopyranoside 26-O-β-D-glucopyranoside on the basis of spectral data and chemical and enzymatic degradation products.

Generally, when crushed between teeth, tomato seeds have a bitter taste. Although an attempt has been made to utilize the tomato seeds for oil or protein resources, no investigation has been made on bitter principles in tomato seeds.

Preliminary experiments were carried out organoleptically with pulverized tomato seeds and showed that the bitter substance was obtained in methanol extracts, and was able to detected by a red color with the Ehrlich reagent (E-reag.) on paper chromatography (PC). The present paper deals with the isolation of the bitter principle in white powders, referred to as TFI, and its chemical structure. This is the first report which shows that natural occurring furostanol saponin contributes to a bitter taste.

Structure of TFI

TFI was isolated by repeated column chromatography of the methanol extracts as a substance with an Rf=0.3 (red with E-reag.) on paper chromatogram using n-butanol-acetic acid-water (4:1:5, upper layer). Enzymatic hydrolysis of TFI with a β-glucosidase afforded prosapogenin-E (Pro-E), which was indicated to be the 25S-spirostanol saponin from the data of IR spectra, νmax at 917 and 892 cm⁻¹ and their relative intensities on the basis of the findings by Wall et al.

A deduction that TFI has furostane structure was supported by the red color reaction with the E-reag. characteristic of furostanol saponin as well as the facile conversion to spirostanol saponin. When acetylation product (I) of TFI was treated as in Fig. 2, yielded δ-hydroxy-γ-methylvaleric acid methyl ester glucoside tetraacetate (VII) which was identified from the NMR data shown in Fig. 3.

Hydrolysis of TFI with 2 N HCl gave a sapogenin, mp 198°C, having an elementary composition of C37H44O9 from elemental analysis and MS, m/e 416 (M⁺); monoacetate, mp 177°C, C39H46O9, was identical with a saturated monohydroxy sapogenin (neotigogenin) belonging to 25S-series by IR νmax at 918 and 892 cm⁻¹ (Fig. 4) and by direct comparison with an authentic neotigogenin [(25S)-5β-spirostan-3β-ol].

From the aqueous layer of the hydrolysate, D-glucose and D-galactose was identified in a molar ratio (3:1). Thus, a possible formula, C51H86O24, was drawn on the basis of the components constituting of one mole each of the aglycone and D-galactose, and three moles of D-glucose. This is actually valid in elemental analysis of TFI itself and its acetate.

Partial hydrolysis of TFI yielded three prosapogenins, called thereafter Pro-I, Pro-II, Pro-III, which were indicative for furostanol saponin structure by a red color reaction with the E-reag, as described above. On the hydrolysis of the prosapogenins, each one was
found to have the following composition by gas chromatography: Pro-I, aglycone and D-glucose (1:1); Pro-II, aglycone, D-glucose and D-galactose (1:1:1); Pro-III, aglycone, D-glucose and D-galactose (1:2:1). On the other hand, enzymatic hydrolysis of TFI using a $\beta$-glucosidase resulted in release of D-glucose and formation of Pro-E (negative for the E-reag.).

Subsequently, permethylated TFI was prepared by the Hakomori method, and after hydrolysis it was analysed by gas chromatography. As the result, three methylated sugars were detected, each of which was isolated by column chromatography and confirmed to be 2,3,4,6-tetra-O-methyl-D-glucopyranose, 3,4,6-tri-O-methyl-D-glucopyranose and 2,3,6-tri-O-methyl-D-galactopyranose, respectively. By the above method, each of fully methylated prosapogenins was
obtained and then subjected to gas chromatography.\textsuperscript{11} Partially methylated sugars identified such are listed in Table II. The results of sugar analysis made clear that TFI has one D-glucose and a straight-chain trisaccharide, D-glucopyranosyl (1→2)-D-glucopyranosyl (1→4)-D-galactopyranose in the molecule. Additionally, a decision of the anomic (α, β) isomers of sugars was performed by the Klyne rule\textsuperscript{12} which had been successfully used for determination of structure of steroid saponins by several workers.\textsuperscript{13,14} According to those literatures, molecular rotation of differences between prosapogenins (Pro-I, Pro-II and Pro-III) and TFI was counted as shown in Table III. Furthermore, based on the fact
TABLE II. METHYL GLYCOSIDES ANALYSIS OF TFI AND PROSAPONEONES BY GAS-LIQUID CHROMATOGRAPHY

<table>
<thead>
<tr>
<th>Glycoside</th>
<th>2,3,4,6-tetra-Methyl-Glc</th>
<th>2,3,4,6-tetra-Methyl-Gal</th>
<th>2,3,6-tri-Methyl-Gal</th>
<th>3,4,6-tri-Methyl-Glc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro-I</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Pro-II</td>
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<td></td>
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<tr>
<td>Pro-III</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro-E</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TFI</td>
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</tbody>
</table>

Conditions: column (G). flow rate (N₂) 30 ml/min.

TABLE III. MOLECULAR ROTATION DIFFERENCE

<table>
<thead>
<tr>
<th></th>
<th>[α]₀</th>
<th>(MeOH-CHCl₃)</th>
<th>[M]₀</th>
<th>[M][α]₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro I</td>
<td>−31</td>
<td>−184</td>
<td>−74</td>
<td></td>
</tr>
<tr>
<td>Pro II</td>
<td>−33</td>
<td>−260</td>
<td>+26</td>
<td></td>
</tr>
<tr>
<td>Pro III</td>
<td>−26</td>
<td>−234</td>
<td>−26</td>
<td></td>
</tr>
<tr>
<td>TFI</td>
<td>−24</td>
<td>−260</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

α-Methyl-d-glucopyranoside: M₀, +309
β-Methyl-d-glucopyranoside: M₀, −66
α-Methyl-d-galactopyranoside: M₀, +379
β-Methyl-d-galactopyranoside: M₀, 0

that each sugar found belongs to d-series, all of the glycosidic linkages questionable were presumed to be β-form.

Thus, a whole structure of the bitter principle in tomato seeds might reasonably be formulated as 5α-furostane-3β,22,26-triol-3-[O-β-d-glucopyranosyl(1→2)-β-d-glucopyranosyl (1→4)-β-d-galactopyranoside] 26-O-β-d-glucopyranoside.

EXPERIMENTAL

Ascending paper chromatography (PC) was carried out on Toyo Roshi No. 50 paper with the following solvent systems: (A) n-butanol-acetic acid-H₂O (4:1:5 upper layer), (B) n-butanol-pyridine-H₂O (6:4:3), (C) n-butanol-ethanol-H₂O (5:1:4). The chromatograms were sprayed with: (D) The Ehrlich reagent, (2g: p-dimethylaminobenzaldehyde-20% HCl) to detect furostanol saponin. (E) Aniline hydrogen phthalate to detect reducing sugar. For gas chromatography, an Hitachi KGL-2B type gas chromatograph fitted with a hydrogen flame ionization detector was used with following glass columns: (F) 1.5% SE 30 on 60/80 mesh Chromsorb W (2 m × 3 mm) at 170°C, (G) 15% poly-butane 1,4 diol-succinate on celite 545 SK (1.2 m × 4 mm) at 175°C. Retention time (R) was measured relative to that of methyl-2,3,4,6-tetra-O-methyl-β-d-glucopyranoside.

IR spectra were measured with a Hitachi EPI-S2 spectrometer. NMR spectra were measured with a PS-100 high resolution NMR spectrometer (100 Mc) or a Hitachi model H-60 high resolution NMR spectrometer (60 Mc). Mass spectra were measured with an RMV-6E Hitachi mass spectrometer. All melting
Isolation of a bitter principle. Using the tomato seeds (59 g) dried at room temperature, a preliminary examination was taken on fractionation in turn with following three solvents; ether (yield 16.3 g), methanol (4.9 g), water (2.5 g) and residues (33 g). By a sensory test, bitter taste was found only in the methanol extract. Thus, tomato seeds (250 g) pulvérized was extracted repeatedly with methanol. After removal of the solvent, the extracts were further extracted with ether. The residues were dissolved in a minimum volume of the solvent, the extracts were further extracted with methanol. After removal of the solvent gave Pro-I in needles. mp 167--175°C, [α]D 25 = -31° (c=1, CH3OH-CHCl3). Found: C, 58.64; H, 8.38. By a similar procedure as in the case of TFI, Pro-I gave crystalline neotigogenin and D-glucose which was identified by PC (solvent-B).

Identification of sapogenin and sugar. TFI (1.0 g) was dissolved in 80 ml of 2 M HCl on a boiling water bath for 1 hr. The hydrolyzate was filtered, and then treated with Ag2CO3 to remove the HCl and filtered again. PC indicated the presence of D-glucose and D-galactose in TFI. Quantitative analysis of sugar components was carried out by gas chromatography. TFI (14.4 mg) was dissolved in a mixture of dioxiane (5 ml), 4 N H2SO4 (4 ml) and an internal standard (1 ml of aqueous solution containing 5 mg of mannitol) and was heated on a boiling water bath for 5 hr. To the reaction mixture, 10 ml of H2O was added, neutralized with BaCO3 and the resulting filtrate was dried in vacuo. After trimethylsilylated by method reported by Sweely, the sample was chromatographed on a column (F). Calibration curve for each sugar was drawn up for a series of volumes of the stock solution.

Partial hydrolysis of TFI. TFI (5 g) was added 1 N H2SO4 (100 ml) and heated under reflux for 20 min. The rapidly cooled solution was filtered, and the residues were dissolved in a minimum volume of methanol and charged onto a silicic acid-celite column (70: 20). Fractions containing only the substance with an Rf = 0.3 were combined and evaporated to dryness in vacuo. The substance obtained in a powder, was purified by precipitation from benzene-methanol to a chromatographically pure and white amorphous powder (3 g). TFI, mp 217--220°C (dec.), [α]D 25 = -24° (c=1, CH3OH-CHCl3) Found: C, 56.83; H, 8.13. Calcd. for C29H46O4: C, 75.94; H, 10.11. IR νmax cm⁻¹: 1728 (OAc), 1750 (OAc), 984, 918, 890, 850 (25S-spirostanol). NMR (in CDCl3): γ 8.98 (d, OAc), 9.05 (C25-CH3), MS; m/e; 458 (M⁺), 139 (base peak).

Molar ratio of D-glucose, D-galactose and aglycone in TFI. Quantitative analysis of sugar components was carried out by gas chromatography. TFI (14.4 mg) was dissolved in a mixture of dioxiane (5 ml), 4 N H2SO4 (4 ml) and an internal standard (1 ml of aqueous solution containing 5 mg of mannitol) and was heated on a boiling water bath for 5 hr. To the reaction mixture, 10 ml of H2O was added, neutralized with BaCO3 and the resulting filtrate was dried in vacuo. After trimethylsilylated by method reported by Sweely, the sample was chromatographed on a column (F). Calibration curve for each sugar was drawn up for a series of volumes of the stock solution.

Hydrolysis of TFI with β-glucosidase. A mixture of
TFI (0.5 g) in 0.1 M phosphate buffer (pH 6.5, 50 ml) and 10 mg of β-glucosidase (Worthington Biochemical Corp.) was kept at 35°C for 20 hr. The precipitates formed were collected by filtration, washed with H₂O and dried over CaCl₂ in vacuo, yielding a white powder (Pro-E). Hydrolyzate of Pro-E refluxed in 1 N HCl afforded crystalline neotigogenin, and D-glucose and D-galactose which were identified by PC. (solvent-B).

Methylation of TFI and hydrolysis of the product. TFI (5 g) was methylated by a method similar to that described by Hakomori.¹⁰ The product was subjected to alumina column chromatography with benzene as a solvent. A pure methylated TFI was obtained (syrup, ca. 3 g). [α]D²⁵ = +84° (H₂O), n-glucose. In addition, the [α]D²⁵ = +70° (H₂O), D-glucose; M-r, yield 190 mg, [α]D²⁴ = +90° (H₂O), n-glucose; M-β, yield 220 mg, followed and on demethylation with BC₁₃₁₅ they led to D-glucose which were identified by PC. (solvent-B). The residue from Fraction-I was further fractionated with H₂O (120ml) and resulted in precipitates. After removal of the precipitates, the filtrate was then treated with Ag₂CO₃, filtered and evaporated in vacuo, giving a syrupy residue. The residue was examined by PC (solvent-C) and three spots of partially methylated sugars were detected; Rf=0.84 (called M-α), 0.71 (M-β), 0.65 (M-γ). The sugar syrup was dissolved in water and charged on a charcoal-celite column (15 : 15 w/w) which was successively eluted with H₂O, 2%, 4%, 6%, 10%, 15%, and 20% ethanol. These eluates were separated into two groups; Fraction-I, eluates with 10% ethanol containing M-β and M-γ, and Fraction-II, eluates with 15, 20% ethanol containing M-α alone. The residue from Fraction-I was further fractionated in 7-g fractions by chromatography on a Avicel (35 g) column using hexane- n-butanol (1 : 1 v/v) saturated with H₂O. Pure M-β and M-γ were found in fraction number 46~50 and in 53~64, respectively.

Partially methylated sugars thus obtained were as follows and on demethylation with BC₁₃₁₅ they led free sugars detectable by PC. M-α, yield 500 mg, [α]D = +90° (H₂O), D-glucose; M-β, yield 220 mg, [α]D = +70° (H₂O), D-glucose; M-γ, yield 190 mg, [α]D = +84° (H₂O), D-galactose. In addition, the individual methylated sugars were converted into their methyl glycosides and examined in GC using a column (G). The Rt value observed were assigned as follow; (1.00, 1.42) for those from M-α is methyl-2,3,4,6-tetra-O-methyl-D-glucopyranoside, (3.08, 3.68) for those from M-β is methyl-3,4,6-tri-O-methyl-D-glucopyranoside and (3.1, 3.70, 4.22, 4.61) for those from M-γ is methyl-2,3,6-tri-O-methyl-D-galactopyranoside, respectively.

Next, both methylated TFI and prosapogenins were methanolyzed by refluxing with 1 N HCl-CH₃OH for 1 hr. After removal of the HCl with Ag₂CO₃, the filtrate was analyzed by the same way as above. The results are summarized in Table II.

Oxidative decomposition product. Peracetate TFI (2 g) taken in 27 ml of acetic anhydride was refluxed at 120°C for 1 hr, after cooling the resultant was added with 5 ml of H₂O, evaporated to dryness in vacuo, yielding yellowish brown residues (ca. 2 g) in which acetyl-β-D-glucopyranoside (VI). The residue (II) was added with 22 ml of glacial acetic acid and 450 mg of sodium acetate, and the resulting solution was cooled to 12°C and with stirring CrO₃ solution (900 mg in 7 ml of 40% AcOH) was added over a period of 15 min and stood for 1 hr at room temperature. The reaction mixture was diluted with 50 ml of H₂O and shaken with CHCl₃ to extract oxidation products. Evaporation yielded a syrup (ca. 2 g) which corresponds to compound-III. The syrup (1.9 g) was added with 2.7 ml of an aqueous solution containing 2.3 g of KOH. On the further addition of 55 ml of t-butanol, the mixture was agitated vigorously at 30°C for 3.5 hr. After t-butanol had been distilled the reaction mixture was extracted with n-butanol to gain compound-IV.¹⁶

5α-Pregnen-16-en-3β-ol-20-one glycoside (IV). The n-butanol layer was chromatographed using a silicic acid-celite (20 : 10 w/w) column in 6-g fractions each with benzene-CH₃OH by increasing the latter. The eluate with benzene-CH₃OH (80 : 20 v/v) afforded IV which crystallized in needles (400 mg) mp 239~243°C (dec.), Found: C, 56.83; H, 8.13. Calcd. for C₃₁H₄₂O₄: C, 57.06; H, 7.83%.

5α-Pregnen-16-en-3β-ol-20-one acetate (V). A mixture of pregnenolone-glycoside (IV, 400 mg) and 2 N HCl-benzene (24~20 ml) was refluxed for 3 hr. After cooling, the mixture were separated into two layers. The aqueous layer was neutralized with Ag₂CO₃, and D-glucose and D-galactose were identified by PC. The benzene layer was evaporated, from which the acetate was prepared by the usual method and crystallized from aqueous alcohol as colorless needles. mp 162~163°C, Found: C, 77.14; H, 9.58. Calcd. for C₃₁H₄₂O₄: C, 77.05; H, 9.56%. MS: m/e 358 (M⁺), IR max cm⁻¹: 1726 1666, (characteristic β-20-ketone) 960, 920, 900.

β-Hydroxy-γ-methylvaleric acid-methyl ester gluco-side tetraacetate (VII). The aqueous layer, after adjusted to pH 3.0 with 3 N HCl, was shaken in turn with n-butanol and CHCl₃, and then neutralized with 2 N NaOH. Evaporation of it afforded a salt mixture. Hydrolysis of the salt with acid or β-glucosidase gave rise to D-glucose which was identified by PC. Besides, 200 mg of the salt were acetylated in a mixture of pyridine (4 ml) and acetic anhydride (3 ml), and poured into ice-water where deposits occurred.
This is likely corresponding to a tetraacetate of the acid glucoside, and so it was taken in methanol (3 ml) and treated with 2% diazomethane in ether (30 ml). After removal of the solvent the residue dissolved in CHCl₃ was subjected to chromatography on silicic acid. By eluting the column with CHCl₃-acetone (8:2 v/v), chromatographically pure syrup was obtained which was assigned to VII on the spectral data. MS: m/e 331, 243, 242, 200, 129, 115. NMR (in CDCl₃): sugar acetate; 4.69–5.1 (3H, m, C–2, C–3, C–4), 5.51 (1H, d, J=7 Hz, C–1), 5.8 (2H, o, C–6), ca. 6.2 (1H, C–5), 7.91, 7.95, 7.99, 8.00 (12H, 4 Acetyl), acid ester; 6.15–6.82 (2H, m, α–C), 6.32 (3H, s, methyl ester), 7.69 (2H, t, α–C) 8–8.83 (3H, m, β and γ–C), 9.09 (3H, d, γ–C methyl).

REFERENCES